Kidney International, Vol. 60 (2001), pp. 543–552

p38 MAPK and MAPK kinase 3/6 mRNA and activities are increased in early diabetic glomeruli

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Background. The p38 mitogen-activated protein kinase (MAPK) pathway is activated by several stress factors, potentially leading to cellular apoptosis and growth. Little is known about the pattern of glomerular p38 MAPK pathway activation during the course of diabetic nephropathy (DN). We examined the activity and expression of the p38 MAPK pathway members, p38 MAPK, MKK3/6, cAMP-responsive element binding protein (CREB), and MAPK phosphatase-1 (MKP-1), in experimental DN in rats over the course of four months.

Methods. Control (C; $N = 16$) and diabetic (DM; $N = 16$) rats were studied. Four rats from each group were sacrificed monthly, and competitive reverse transcription-polymerase chain reaction and Western blot were performed with microdissected and sieved glomeruli, respectively.

Results. Glomerular p38 MAPK mRNA expression was significantly higher in DM than C ($P < 0.01$) throughout the four-month period. Western blot revealed an average 3.1-fold increase in p38 MAPK protein throughout the study period ($P < 0.05$). However, p38 MAPK activity was significantly increased only in one- and two-month diabetic glomeruli. Glomerular MKK3/6 and CREB mRNA as well as activity were significantly increased only in one- and two-month DM compared with C. MKP-1 mRNA showed a similar pattern.

Conclusions. Glomerular p38 MAPK activity was increased in early DN. Parallel to this, we also showed, to our knowledge for the first time, that there were increased MKK3/6 and CREB activities and mRNA expression. This activated p38 MAPK pathway in diabetic glomeruli may, in part, play a role in the pathogenesis of early hypertrophy and extracellular matrix accumulation.

The molecular and cellular mechanisms responsible for diabetic nephropathy (DN) remain incompletely resolved. While studies indicate involvement of hyperglycemia via the stimulation of growth factor-induced cellular hypertrophy [1, 2], increased production of extracellular matrix protein (ECM) [3, 4], and decreased production of matrix-degrading proteinases [5, 6], the underlying signal transduction mechanisms mediating these processes have been less well explored.

Numerous studies reveal protein kinase C (PKC) activation in diabetic glomeruli [7] and in mesangial cells cultured under high-glucose conditions [8, 9]. PKC propagates the physiologic responses of receptor-ligand interactions via an array of downstream signals, such as mitogen-activated protein kinases (MAPKs). Ultimately, these transmitted signals regulate the transcription of genes responsible for key cellular responses such as proliferation, differentiation, and apoptosis [10–12].

The MAPK family includes the extracellular signal-regulated kinase-1/2 (ERK1/2) or p42/44 MAPK [12, 13], stress-activated c-Jun N-amino terminal kinase (JNK/stress-activated protein kinase or SAPK) [11, 12, 14], p38 MAPK [15–17], and big MAPK (ERK 5) [18]. The classic MAPK ERK1/2 plays a pivotal role in growth factor-induced mitogenesis, differentiation, and cellular transformation. ERK1/2 is activated through a Ras-dependent signal transduction pathway by hormones and growth factors. It leads to cellular proliferation and differentiation by stimulating transcription factors that induce the expression of c-fos and other growth-responsive genes [10, 12, 13]. JNK/SAPK and p38 MAPK represent two independent parallel MAPK pathways that are activated in response to stress signals such as proinflammatory cytokines [11, 14–16, 19], ultraviolet irradiation [14], osmolality changes [15], and oxidants [20], leading to alterations in cell growth, prostanoid production, and other cellular dysfunction [21, 22].

Since hyperosmolality and oxidant stress characterize the diabetic state, p38 MAPK has been posited to mediate the pathogenesis of diabetic complications. Increased p38 MAPK activity has been observed in the aorta of diabetic rats and in vascular smooth muscle cells cultured under high-glucose conditions [23, 24]. Increased mesangial cell p38 MAPK activity has been identified in associ-

Key words: hypertrophy, extracellular matrix, p38 MAPK, renal glomeruli, CREB.

Received for publication December 29, 2000
and in revised form March 8, 2001
Accepted for publication March 12, 2001

ation with high glucose culture conditions [25] and with mechanical strain [26], the latter also activating ERK1/2. However, the p38 MAPK pathway has heretofore not been extensively studied in diabetic glomeruli, and little is known about time-course changes of MAPK in experimental DN in vivo. Furthermore, there are no reports regarding the regulation of the upstream kinase activators of p38 MAPK, namely MKK3/6 under diabetic conditions.

This study examined the p38 MAPK pathway in glomeruli isolated from streptozotocin-induced diabetic rats one, two, three, and four months after diabetes induction. Besides p38 MAPK, we also studied (*1*) MKK3 and MKK6, which are upstream activators of p38 MAPK [27, 28]; (*2*) cAMP-responsive element binding protein (CREB), a transcription factor that could be activated by protein kinase A (PKA) as well as p38 MAPK [29–31]; and (*3*) MAPK phosphatase-1 (MKP-1), a phosphatase that dephosphorylates tyrosine and threonine residues of p38 MAPK, resulting in deactivation of p38 MAPK [32]. In the present study, we have examined not only the activities of these key signaling kinases and target transcription factor, but also their mRNA expression during the course of DN. We also correlated these with the expression of fibronectin and transforming growth factor-β (TGF-β), key markers of DN.

METHODS

Animals

All animal studies were conducted under an approved institutional protocol. Sprague-Dawley rats weighing 220 to 250 g were injected intraperitoneally with either diluent [$N = 16$, control (C)] or 65 mg/kg streptozotocin (STZ) [$N = 16$, diabetes (DM)]. Blood glucose levels were measured on the third day after STZ injection to confirm the development of diabetes. Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the four-month study period. Four rats from each group were sacrificed at the end of one, two, three, or four months. Sieved glomeruli obtained from the one- to four-month rats were used for Western blots, while microdissected glomeruli from the one-, two-, and four-month rats were used for RNA extraction and polymerase chain reactions (PCRs). Blood glucose levels of the control rats at one, two, three, and four months were as follows: 103.1 ± 4.5, 106.5 ± 2.4, 102.2 ± 4.8, 95.4 ± 3.8, and 104.5 ± 6.9, respectively. Blood glucose levels in the diabetic rats were 438.8 ± 18.6 at three days post-STZ, 453.0 ± 18.8 at one month, 441.4 ± 23.2 at two months, 447.5 ± 15.2 at three months, and 494.3 ± 17.8 at four months.

Western blot analysis

Sieved rat glomeruli were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mmol/L Tris-HCl, pH 6.8, 10% (vol/vol) glycerol]. The lysate was centrifuged at 10,000 × *g* for 10 minutes at 4°C, and the supernatant was stored at −70°C until all rats were sacrificed. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of 50 μg protein were treated with Laemmli sample buffer, heated at 100°C for five minutes, electrophoresed, and transferred to Hybond-ECL membranes (Amersham Life Science, Inc., Arlington Heights, IL, USA). Blocked membranes were incubated overnight at 4°C with a 1:1000 dilution of polyclonal antibody to p38 MAPK, phospho-specific p38 MAPK, phospho-specific MKK3/6, or phospho-specific CREB (New England Biolabs, Inc., Beverly, MA, USA). This was followed by incubation with horseradish peroxidase-linked sheep anti-mouse IgG (Amersham) at 1:1000 dilution. Developing was with a chemiluminescent agent (ECL; Amersham).

Microdissection, total RNA extraction, and reverse transcription

Glomerular microdissection, total RNA extraction, and reverse transcription (RT) were performed as described recently [33].

Semiquantitative competitive PCR

Competitor cDNAs were used as internal standards in semiquantitative PCR evaluation of GAPDH, p38 MAPK, MKK3, MKK6, CREB, fibronectin, MKP-1, and TGF-β mRNAs. Each competitor was designed to contain the same base-pair sequence as the target cDNA, but had a portion deleted so that the competitor PCR-generated fragment could be easily distinguished electrophoretically by size. The primers used are summarized in Table 1. cDNA from 1/10 of a glomerulus per reaction tube was used for GAPDH and fibronectin, 1/5 of a glomerulus for p38 MAPK, and 1/2 glomerulus for the remainder. PCR was performed using the test or wild-type cDNA, Taq-Gold polymerase (1.5 U for GAPDH, p38 MAPK, and fibronectin; 2.5 U for the remainder), 20 μmol/L dNTP, sense and antisense primers (25 pmol for GAPDH, p38 MAPK, and fibronectin; 30 pmol for the remainder), and specific dilution of competitor in a volume of 50 μL containing 1 × PCR buffer. The PCR conditions were denaturation at 95°C for 45 seconds, annealing at corresponding temperature for 45 seconds, and extension at 72°C for two minutes (Table 1). Initial heating at 95°C for nine minutes and final extension at 72°C for seven minutes was performed for all PCRs. The RT-PCR products were separated by electrophoresis. Band densities were analyzed by laser densitometry (Helena Laboratories, Beaumont, TX, USA), and the ratio of wild type to competitor was used for analyses.

Table 1. Primers sequences and PCR conditions

	Sequence (5′ → 3′)	Annealing temperature °C	No. of cycles
GAPDH			
Sense	GACAAGATGGTGAAGGTCGG	62	36
Antisense	CATGGACTGTGGTCATGAGC		
P38 MAPK			
Sense	CGAAATGACCGGCTACGTGG	65	40
Antisense	CACTTCATCGTAGGTCAGGC		
MKK3			
Sense	GCCTTCCAATGTCCTCATCAAC	62	48
Antisense	GGATTCTTCCTGAGGCACTGG		
MKK6			
Sense	GGTGGAGAAGATGCGTCACG	62	48
Antisense	CCAGGTAGCCGCTGATTCC		
MKP-1			
Sense	GCTTCAGCACCATCGTGC	63	45
Antisense	GCATGGTAAGCACTGCCC		
CREB			
Sense	GGGAAATCCTTTCAAGGAGGC	61	42
Antisense	CGACATTCTCTTGCTGCTTCC		
Fibronectin			
Sense	GCAAGCCTGAACCTGAAGAGACC	62	38
Antisense	CCTGGTGTCCTGATCATTGCATC		
TGF-β			
Sense	CGAGGTGACCTGGGCACCATCC	62	42
Antisense	GCTCCACCTTGGGCTTGCGACC		

Abbreviations are in the Appendix.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analysis was performed using the statistical package SPSS for Windows version 7.51 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the Student *t* test or analysis of variance (ANOVA) for multiple comparisons.

RESULTS

A time-course study was performed wherein the activities as well as mRNA expression were simultaneously examined of p38 MAPK, MKK3/6 (upstream activators of p38 MAPK), and CREB, in addition to MKP-1 mRNA in glomeruli isolated from control rats versus rats that had been diabetic for one to four months. To correlate these parameters to the progression of DN, mRNA expression of the key ECM protein fibronectin as well as transforming growth factor-β (TGF-β) also were evaluated.

p38 MAPK activity, protein, and mRNA expression

We first examined whether p38 MAPK activity, protein, as well as mRNA expression were altered in the one- to four-month time period after STZ injection. Figure 1 shows a representative Western blot of equal amounts of protein from lysates of sieved glomeruli from the control and DM rats at one to four months. The blot was first probed with an antibody to phospho-specific p38 MAPK (upper panel), stripped, and then probed with an antibody that recognizes total p38 MAPK (all isoforms, lower panel). Interestingly, phospho-specific p38 MAPK levels, which represent p38 MAPK activity, were greater in the diabetic rats relative to the corresponding control rats at one and two months ($P < 0.01$ at each time), but decreased to control levels thereafter. In contrast, although total p38 levels (lower panel) were unchanged from one to four months in each group alone, they were significantly elevated (3-fold) in the diabetic glomeruli compared with control at all four time points ($P < 0.05$). The ratios of phospho-p38 to total p38 MAPK levels were significantly greater in DM glomeruli only at one and two months ($P < 0.01$). Equal protein loading was confirmed by Coomassie Blue staining of the gel.

To evaluate whether this increase in p38 MAPK expression in DM glomeruli was also regulated at the mRNA level, total RNA from microdissected glomeruli from the one-, two- and four-month study were used. We developed a new competitive PCR using primers derived from the sequence of rat p38 MAPK [15]. The p38 MAPK mRNA ratios (wild-type:competitor) were significantly greater in DM rats than the corresponding C and were over twofold greater at all three time points studied (Fig. 2). In contrast, glomerular GAPDH mRNA ratios (wild-type:competitor) in the DM glomeruli did not differ from those in the C group (data not shown). p38 MAPK mRNA ratios remained significantly higher in DM relative to corresponding C after correction for GAPDH mRNA ratios (Fig. 3).

Thus, total p38 MAPK protein and mRNA expression are increased in experimental DN, but a new level of

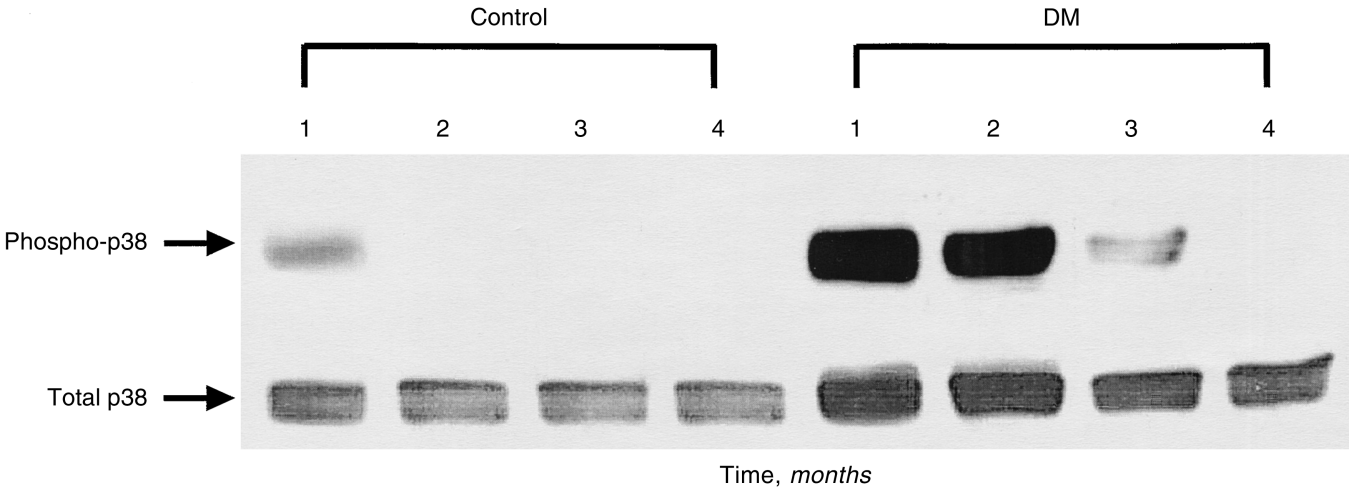

Fig. 1. Western blot of glomerular total and phospho-specific p38 mitogen-activated protein kinase (MAPK) in control and diabetic (DM) rats (representative of four blots). Total p38 MAPK protein expression (lower panel) was significantly higher in DM than control throughout the four months (average 3.1-fold increase by densitometry, $P < 0.05$). On the other hand, activated phospho-specific p38 MAPK protein (upper panel) showed a significant increase only in one- and two-month DM glomeruli ($P < 0.001$).

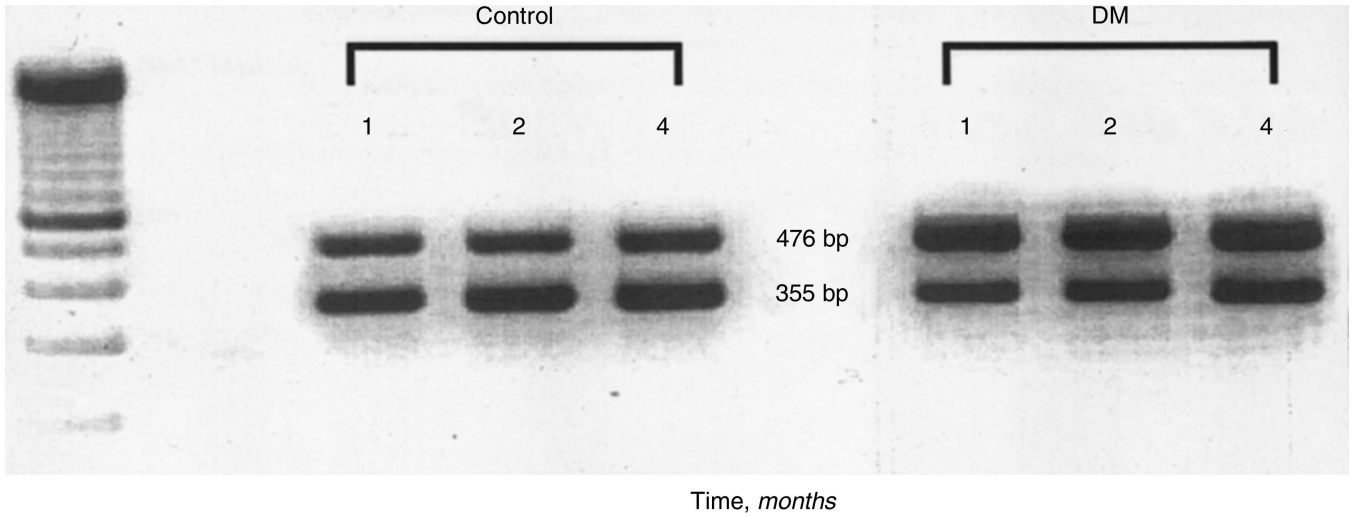

Fig. 2. Glomerular p38 MAPK mRNA RT-PCR in one-, two-, and four-month control and DM rats. Each lane represents a competitive RT-PCR reaction (40 cycles) of RNA from microdissected glomeruli. A fixed amount of p38 MAPK wild-type cDNA (476 bp) from the equivalent of 0.2 glomeruli from each rat and fixed amount of p38 MAPK competitor cDNA (355 bp) were used. All data are expressed as the ratio of wild-type:competitor optical density after densitometric analyses. Data shown are representative of four gels.

regulation of p38 MAPK enzyme activity is operative in the early versus later stages of DN.

MKK3/6 activity and mRNA expression

The activity and mRNA expression of the upstream kinase activators of p38 MAPK, namely, MKK3/6, were examined. The blot shown in Figure 1 was stripped and reprobed with an antibody that recognizes both phospho-specific MKK3 and MKK6 (activated forms of these kinases). This representative Western blot seen in Figure 4 shows that phospho-specific MKK3/6 levels were also increased in one- and two-month diabetic glomeruli relative to control, but not thereafter (1.8- and 2.1-fold increments at one and two months, respectively, $P < 0.05$). This trend was similar to phospho-specific p38 MAPK (Fig. 1, upper panel).

Since DM seemed to up-regulate MKK3/6 activity, we wanted to determine whether this was related to mRNA expression. Here again, we successfully developed a specific competitive PCR approach to evaluate MKK3 and MKK6 mRNA levels. Glomerular MKK3 as well as MKK6 mRNA ratios (wild-type:competitor) were significantly higher in the one- and two-month DM rats compared with corresponding C rats, but unlike p38 MAPK

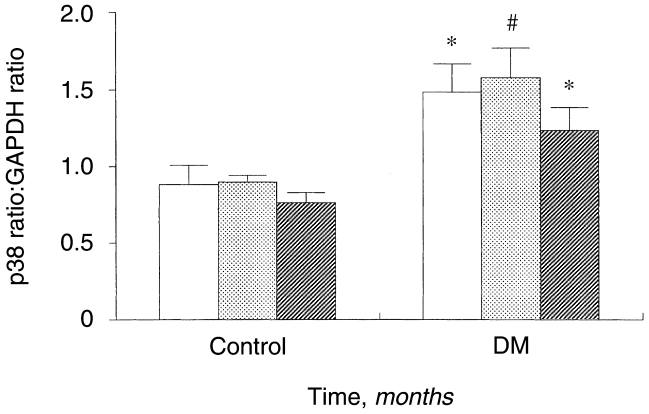

Fig. 3. Glomerular p38 MAPK mRNA ratio:GAPDH mRNA ratio of the rats. Ratio equals the optical density of wild-type band to the optical density of the competitor band. p38 MAPK mRNA ratios were then corrected for the GAPDH mRNA ratio. These ratios were significantly higher in DM compared with corresponding control rats throughout the four-month study. Symbols are: (□) 1 month; (▒) 2 months; (▨) 4 months; $*P < 0.05$ vs. control; $\#P < 0.01$ vs. control.

mRNA, the MKK3/6 mRNAs returned to control levels at four months. After correcting for GAPDH mRNA ratios in each sample, there remained a significant, nearly twofold increase in MKK3 and MKK6 mRNAs at one- and two-month time periods, but not at four months (Figs. 5 and 6).

These results demonstrate, to our knowledge for the first time, the regulation of MKKs under diabetic conditions, and also suggest a new level of potential transcriptional regulation of p38 MAPK family signaling molecules in DN.

CREB activity and mRNA expression

To determine whether activation of the p38 MAPK pathway could induce parallel increases in the activity of a p38 MAPK target transcription factor, we examined the activity and mRNA expression of CREB due to its role in fibronectin transcription [34, 35]. Glomerular protein lysates were subjected to immunoblotting using an antibody to phospho-specific (activated) CREB. The representative blot in Figure 7 shows that CREB activity was significantly increased in the one- and two-month DM samples relative to C ($P < 0.005$). However, similar to p38 MAPK and MKK3/6 activities, CREB activity also declined at three and four months.

We then developed a specific competitive PCR technique to evaluate CREB mRNA levels. Glomerular CREB mRNA ratios (wild-type:competitor) were greater in the DM glomeruli compared with C at one and two months, but not at four months. This trend was parallel to that observed with MKK3/6 mRNAs, but different from p38 MAPK mRNA, which remained elevated even at four months. After correcting for GAPDH mRNA ratios, there were significant 1.7- and 1.8-fold increases in CREB mRNA expression at one and two months, respectively (Fig. 8). These results show for the first time that increased CREB activity in early DN may be a consequence of increased activity of its upstream kinase activators as well as increased CREB mRNA expression.

MKP-1 mRNA expression

To determine whether the fall in p38 MAPK and MKK3/6 activities in later time periods was due to the action of a phosphatase, the expression of MKP-1 mRNA also was examined by competitive PCR. Glomerular MKP-1 mRNA ratios (wild-type:competitor) were significantly greater in DM versus C only at one and two months, but not at four months, both before (data not shown) and after correction for GAPDH ratios (Fig. 9). Hence, the fall-off in kinase activities after two months could not be attributed to changes in MKP-1 mRNA levels.

Fibronectin and TGF-β mRNA expression

To correlate these observations to evolving DN, the mRNA expression of the key matrix protein, fibronectin, as well that of the growth factor, TGF-β, were examined using semiquantitative competitive PCR. The glomerular fibronectin mRNA ratio (wild-type:competitor) was significantly higher in the DM relative to the corresponding C rats throughout the four-month period before (data not shown) and after correction for GAPDH mRNA levels ($P < 0.005$; Fig. 10).

Similarly, glomerular TGF-β mRNA ratios (wild-type: competitor) were significantly greater in the DM rats relative to corresponding C rats throughout the four-month period before (data not shown) and after correction for GAPDH mRNA levels ($P < 0.01$; Fig. 11).

Thus, glomerular mRNA levels of all the factors examined, p38 MAPK, MKK3/6, CREB, MKP-1, fibronectin, and TGF-β (except the GAPDH control), were significantly higher in DM compared with C rats at one and two months after STZ or diluent injections, respectively. This suggests a p38 MAPK-regulated mechanism leading to fibronectin expression in early DN. However, a more complex pattern emerges by three to four months, with increased p38 MAPK mRNA and protein expression (without corresponding p38 MAPK activity), normalization of mRNAs and activities of MKK3/6 and CREB, and nevertheless, the maintenance of increased fibronectin and TGF-β mRNA expression.

DISCUSSION

Diabetic nephropathy is characterized by glomerular hypertrophy and ECM accumulation [36, 37]. Since p38 MAPK is activated by cellular stressors and has been implicated in the pathogenesis of cardiac and vascular smooth muscle cell hypertrophy [38, 39], TGF-β–induced gene expression and TGF-β formation [40, 41], we fo-

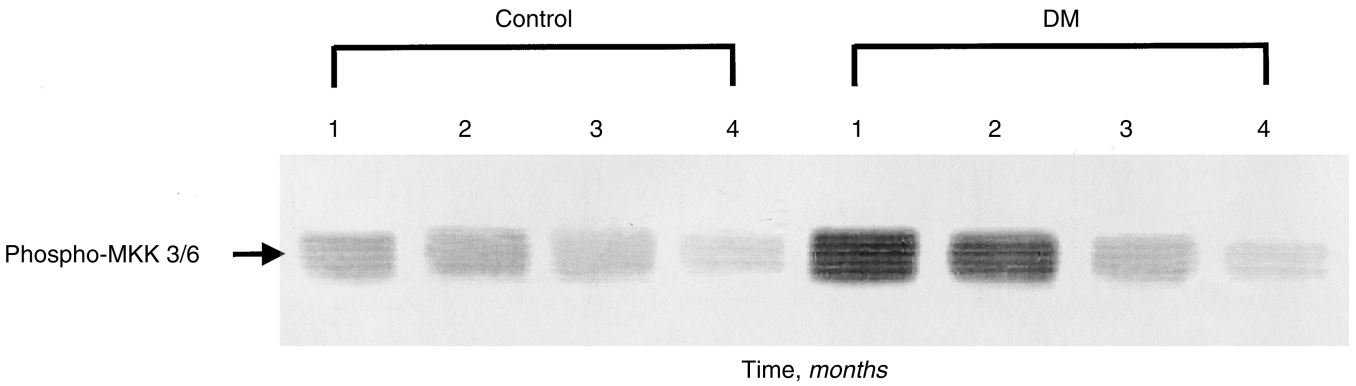

Fig. 4. Western blot of phospho-specific MAPK kinase 3/6 (MKK3/6) with control and DM glomeruli (representative of four blots). There were 1.8- and 2.1-fold increases in MKK3/6 activity in one- and two-month DM compared with the corresponding control rats, respectively ($P < 0.05$).

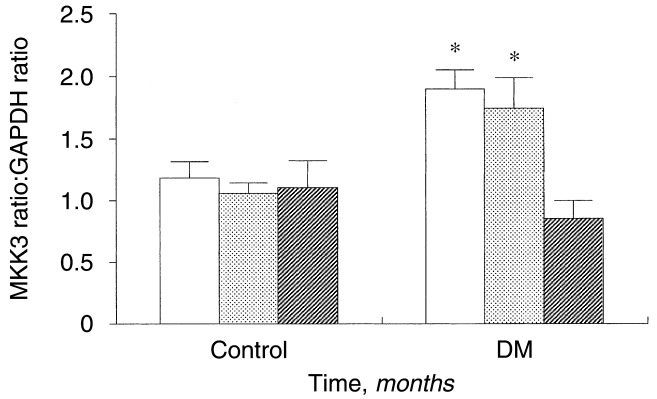

Fig. 5. Glomerular MKK3 mRNA ratio:GAPDH ratio of the rats. Glomerular MKK3 mRNA expression was significantly higher in one- and two-month DM rats than in corresponding control rats and returned to control level after four months. Symbols are: (□) 1 month; (▒) 2 months; (▓) 4 months; *$P < 0.05$ vs. control. MKK3 mRNA expression showed a similar pattern as the phospho-specific MKK3/6 protein expression (Fig. 4).

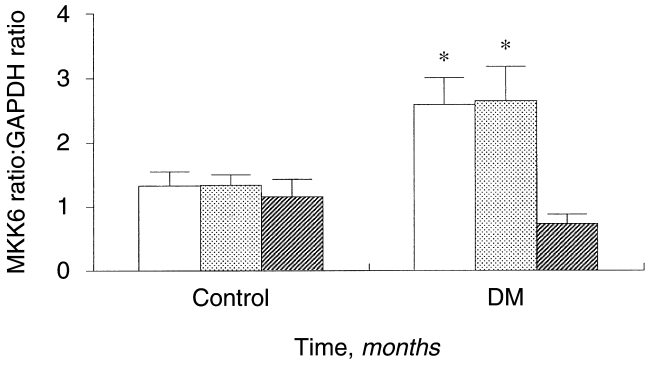

Fig. 6. Glomerular MKK6 mRNA ratio:GAPDH ratio. Glomerular MKK6 mRNA expression showed a similar pattern as MKK3 mRNA. There was a significant increase in MKK6 mRNA in one- and two-month DM rats compared with corresponding control rats. Symbols are: (□) 1 month; (▒) 2 months; (▓) 4 months; *$P < 0.05$ vs. control.

cused on the p38 MAPK pathway rather than other MAPKs to discern the effector pathways that may mediate DN. We demonstrate, to our knowledge for the first time, that glomerular p38 MAPK activity is increased in early DN and that the activation of p38 MAPK is associated with and likely due to increased MKK3 and MKK6 at the mRNA and activity levels.

p38 MAPK is a member of the MAPK family and is known as a "stress-activated kinase" along with the JNK [14–17]. p38 MAPK is strongly activated by environmental stressors, including osmolality changes [15], oxidants [20], and proinflammatory cytokines [19], leading to cellular growth, differentiation, and apoptosis [21, 38]. Recent work has shown that high ambient glucose concentrations increased p38 MAPK activity in cultured vascular smooth muscle cells via PKC-dependent and -independent pathways [23, 24]. Increased p38 MAPK protein and activity were also observed in aortas of diabetic rats [23]. Other in vitro studies also demonstrated that short-term exposure to high glucose activated p38 MAPK in cultured rat vascular smooth muscle cells [24] and mesangial cells [25]. However, not all investigators report increased p38 MAPK activity in cultured mesangial cells treated with high glucose [42]. These inconsistent findings may result from different culture conditions and/or glucose concentrations utilized in these experiments. In our current study, we observed increased p38 MAPK protein expression as well as activity in early diabetic glomeruli, a finding that is consistent with the aforementioned study in diabetic aorta [23].

The activity of the MAPK family including p38 MAPK is determined by a dynamic balance between phosphorylation and dephosphorylation, resulting from dual specific protein kinases (MAPK kinase, MKK, or MEK) and phosphatase, respectively [27, 28, 32]. Among numerous MAPK kinases, MKK3 and MKK6 catalyze the phosphorylation of p38 MAPK specifically and MKK4/SEK1 mediates the activation of both p38 MAPK and JNK [27, 28]. MKK3 and/or MKK6 are activated by factors such as

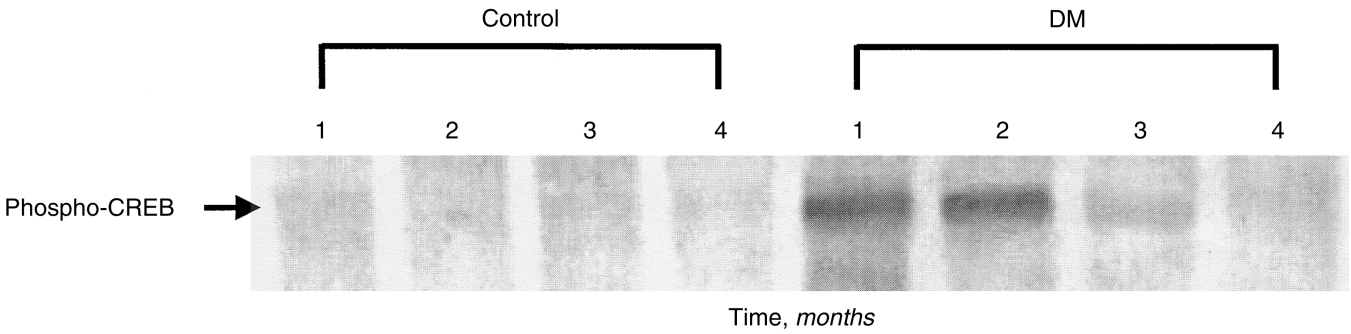

Fig. 7. Western blot of glomerular phospho-specific cAMP-responsive element binding protein (CREB) in control and DM rats (representative of three blots). Phospho-CREB levels were significantly higher in the one- and two-month DM glomeruli than in the corresponding control glomeruli ($P < 0.005$).

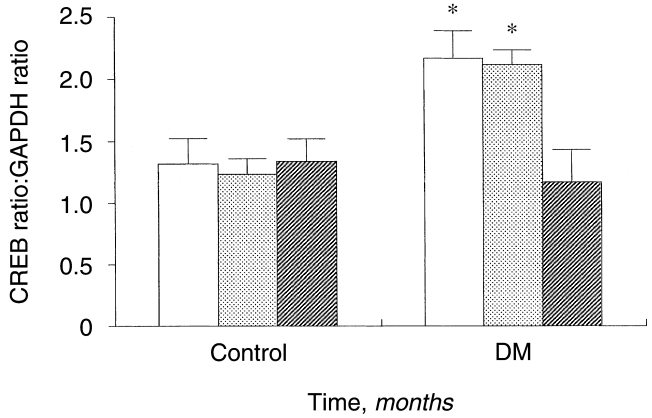

Fig. 8. Glomerular CREB mRNA ratio:GAPDH ratio of the rats. CREB mRNA expression at one and two months was significantly higher in DM than control rats, but at four months, there was no significant difference between the two groups. Symbols are: (□) 1 month; (▒) 2 months; (▓) 4 months; *$P < 0.05$ vs. control.

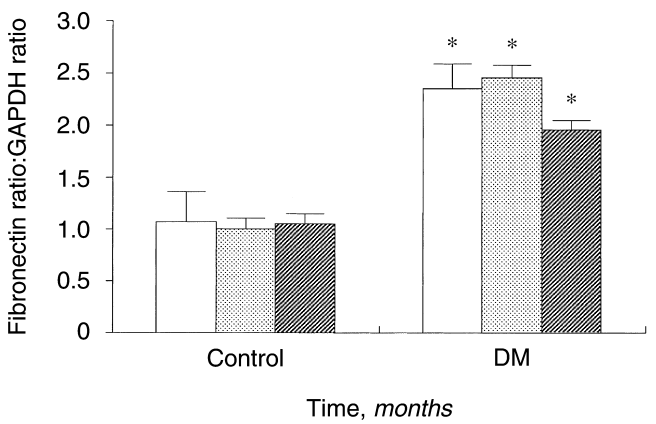

Fig. 10. Glomerular fibronectin mRNA ratio:GAPDH ratio of the rats. Fibronectin mRNA expression was significantly increased in DM compared with corresponding control glomeruli throughout the four-month study period. Symbols are: (□) 1 month; (▒) 2 months; (▓) 4 months; *$P < 0.005$ vs. control.

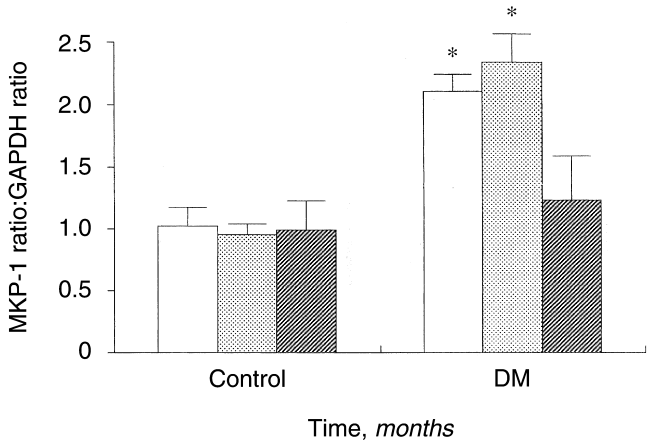

Fig. 9. Glomerular MKP-1 mRNA ratio:GAPDH ratio of the rats. MAPK phosphatase-1 (MKP-1) mRNA expression was significantly higher in one- and two-month DM than corresponding control rats. Symbols are: (□) 1 month; (▒) 2 months; (▓) 4 months; *$P < 0.005$ vs. control.

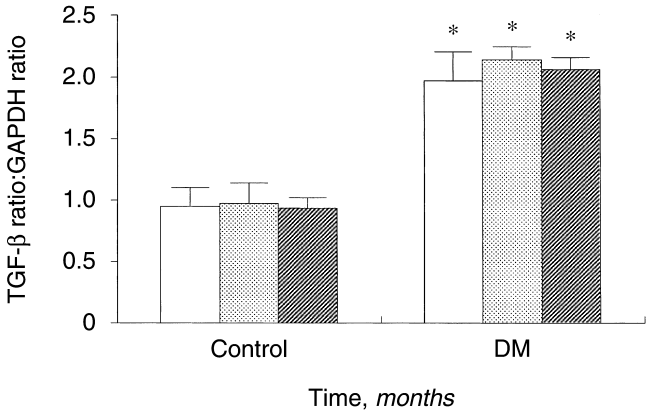

Fig. 11. Glomerular transforming growth factor-β (TGF-β) mRNA ratio:GAPDH ratio of the rats. Glomerular TGF-β mRNA expression was significantly increased in DM rats compared with corresponding control rats throughout the four-month study period. Symbols are: (□) 1 month; (▒) 2 months; (▓) 4 months; *$P < 0.01$ vs. control.

osmotic shock [43] and lipopolysaccharide (LPS) [44]. Although some studies have demonstrated p38 MAPK activation under diabetic conditions [23, 25], little is known about the upstream activators of p38 MAPK in this setting. Haneda et al observed activation of MEK1, an upstream protein kinase of ERK1, in diabetic glomeruli and mesangial cells under high glucose conditions, but did not assess the p38 MAPK pathway [45]. Our study demonstrates, for the first time to our knowledge, that MKK3 and MKK6 activity as well as mRNA expression is increased in early diabetic glomeruli, presumably resulting in p38 MAPK activation. We also show for the first time that p38 MAPK mRNA expression is increased.

Mitogen-activated protein kinase phosphatase-1 is a member of the rapidly growing, dual-specific protein tyrosine phosphatase family that dephosphorylates and inactivates various members of the MAPK family [32, 46]. Initial reports regarding MKP-1 demonstrated that MKP-1 was associated with ERK both in vivo and in vitro [46], but recent investigations suggest that MKP-1 exhibits a broad substrate specificity, being able to inactivate both JNK and p38 MAPK [20, 44]. MPK-1 is principally regulated at the transcriptional level [47], as evidenced by very low to undetectable mRNA expression in quiescent cells and a rapid induction by multiple signaling molecules, including growth factors and angiotensin II [32, 46, 48–52]. Most previous in vitro studies have suggested that MKP-1 is implicated in a feedback loop serving to inactivate MAPK after stimulation by mitogens or cellular stress [20, 50].

Even though high glucose is known to exert cellular stress, the effect of hyperglycemia on MKP-1 expression remains controversial. An in vitro study of vascular smooth cells revealed that 12 to 24 hours of incubation in high glucose (25 mmol/L) decreased MKP-1 mRNA and protein [51]. MKP-1 protein expression was also suppressed in mesangial cells grown under high glucose (30 mmol/L) for five days, resulting in activation of ERK [52]. However, Kang et al observed that MKP-1 protein was increased in mesangial cells after one hour of exposure to high glucose (40 mmol/L), sustained for 24 hours, and declined to control levels after 48 hours [25]. The reasons for the discrepant effects of high glucose on MKP-1 are unclear, but may result from differences in cell type, use of pooled samples, duration of high glucose exposure, and glucose concentrations used. Our study found that the time course of glomerular MKP-1 mRNA expression coincided with activation of p38 MAPK. These results implicate MKP-1 induction in early diabetic glomeruli as a negative feedback loop to dephosphorylate activated MAPK including p38 MAPK, rather than a pathogenetic mechanism for p38 activation.

Once p38 MAPK is activated, it phosphorylates several transcription factors at serine and threonine residues including CREB and activating transcription factor-1 (ATF-1), ATF-2, Elk-1, CHOP, and myocyte enhancer factor-2C (MEF-2C), thereby regulating gene expression [19, 27, 29–31, 53–55]. In addition to these factors, the p38 MAPK also activates other protein kinases. Among these downstream kinases, mitogen- and stress-activated protein kinase-1 (MSK-1), MAPK-activated protein kinase-2 (MAPKAPK-2), and ribosomal S6 kinase-B (RSK-B) have been implicated in the phosphorylation of CREB after activation by the p38 MAPK pathway [30, 31]. CREB can lead to gene expression by binding to the cAMP response element (CRE). Since the fibronectin promotor contains a CRE located −170 bp [56], activated CREB can bind to this CRE, leading to fibronectin mRNA expression. Kreisberg et al have demonstrated that activation of PKC by high glucose plus TGF-β or phorbol 12 myristate 13-acetate (PMA) induced CREB activation and fibronectin transcription in mesangial cells in vitro [34]. Nahman et al have demonstrated the involvement of CRE elements in angiotensin II-induced fibronectin expression in mesangial cells [35]. We now report increased fibronectin mRNA expression along with increased CREB mRNA and activity in early diabetic glomeruli in vivo.

In these experiments, the p38 MAPK pathway was functionally activated in early diabetic glomeruli but returned to the control levels with time, a new in vivo finding that is in agreement with most previous in vitro studies. Although the mechanism of this deactivation at later time periods is presently unclear, it seems that more proximal signal transduction molecules than MKK3 and MKK6 may be involved. Glomerular fibronectin mRNA expression was increased not only in early diabetic rats but also after three months, even when the p38 MAPK pathway was already deactivated. These findings suggest that fibronectin mRNA expression is not totally dependent on the MKK3/6-p38MAPK-CREB axis. TGF-β is known to play an important role in the synthesis of ECM molecules in DN [57, 58]. Previous studies have demonstrated that TGF-β activates at least two independent pathways, the TGF-β–activated kinase 1 (TAK1)-MKK6-p38MAPK pathway and the Smad-mediated pathway [59, 60]. Even though TGF-β activates the p38 MAPK pathway, a recent study showed that dominant negative TAK1 or dominant negative MKK6 fails to inhibit TGF-β–induced signal transduction in C2C12 myoblast cells due to an intact Smad-mediated pathway [40]. In our study, glomerular TGF-β mRNA expression was significantly higher in DM rats compared with C rats throughout the four-month study period similar to fibronectin. This suggests that an intact TGF-β-Smad pathway may mediate the sustained increase in fibronectin mRNA after three to four months when the MKK3/6-p38 MAPK-CREB pathway is already deactivated.

In summary, p38 MAPK activity was increased in early diabetic glomeruli, and this increase was associated with

and likely mediated by increased MKK3/6 activity and mRNA expression. Furthermore, the activity of CREB, a transcription factor activated by p38 MAPK and associated with fibronectin transcription, was also increased in glomeruli from one- and two-month diabetic rats, coinciding with the p38 MAPK activity. This activated p38 MAPK pathway in diabetic glomeruli may, in part, play a role in the pathogenesis of early hypertrophy and extracellular matrix accumulation.

ACKNOWLEDGMENTS

This work was supported by grants from the Juvenile Diabetes Foundation International and the National Institutes of Health. The authors thank Ms. Linda Lanting for technical assistance.

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APPENDIX

Abbreviations used in this article are: ATF-1, activating transcription factor-1; C, control; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; DM, diabetic; DN, diabetic nephropathy; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ERK1/2, extracellular signal-regulated kinase-1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK/SAPK, stress activated c-Jun N-amino terminal kinase/stress-activated protein kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKP-1, MAPK phosphatase-1; MAPKAPK-2, MAPK-activated protein kinase-2; MEF-2C, myocyte enhancer factor-2C; MSK-1, mitogen- and stress-activated protein kinase-1; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; RSK-B, ribosomal S6 kinase-B; SDS, sodium dodecyl sulfate; STZ, streptozotocin; TAK1, transforming growth factor-β–activated kinase 1; TGF-β, transforming growth factor-β.

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